Hepatic Recruitment of the Inflammatory Gr1$^+$ Monocyte Subset Upon Liver Injury Promotes Hepatic Fibrosis

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In addition to liver-resident Kupffer cells, infiltrating immune cells have recently been linked to the development of liver fibrosis. Blood monocytes are circulating precursors of tissue macrophages and can be divided into two functionally distinct subpopulations in mice: Gr1^{hi} (Ly6C^{hi}) and Gr1^{lo} (Ly6C^{lo}) monocytes. The role of these monocyte subsets in hepatic fibrosis and the mechanisms of their differential recruitment into the injured liver are unknown. We therefore characterized subpopulations of infiltrating monocytes in acute and chronic carbon tetrachloride (CCl_4)-induced liver injury in mice using flow cytometry and immunohistochemistry. Inflammatory Gr1^{hi} but not Gr1^{lo} monocytes are massively recruited into the liver upon toxic injury constituting an up to 10-fold increase in CD11b$^+$F4/80$^+$ intrahepatic macrophages. Comparing wild-type with C-C chemokine receptor (CCR2)-deficient and CCR2/CCR6–deficient mice revealed that CCR2 critically controls intrahepatic Gr1^{hi} monocyte accumulation by mediating their egress from bone marrow. During chronic liver damage, intrahepatic CD11b$^+$F4/80$^+$Gr1$^+$ monocyte-derived cells differentiate preferentially into inducible nitric oxide synthase–producing macrophages exerting proinflammatory and profibrogenic actions, such as promoting hepatic stellate cell (HSC) activation, T helper 1–T cell differentiation and transforming growth factor β (TGF-β) release. Impaired monocyte subset recruitment in *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice results in reduced HSC activation and diminished liver fibrosis. Moreover, adoptively transferred Gr1^{hi} monocytes traffic into the injured liver and promote fibrosis progression in wild-type and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice, which are otherwise protected from hepatic fibrosis. Intrahepatic CD11b$^+$F4/80$^+$Gr1$^+$ monocyte-derived macrophages purified from CCl_4-treated animals, but not naïve bone marrow monocytes or control lymphocytes, directly activate HSCs in a TGF-β–dependent manner *in vitro*. *Conclusion:* Inflammatory Gr1$^+$ monocytes, recruited into the injured liver via CCR2-dependent bone marrow egress, promote the progression of liver fibrosis. Thus, they may represent an interesting novel target for antifibrotic strategies. (HEPATOLOGY **2009;50:261-274.**)

Liver fibrosis as the common end-stage of chronic liver diseases is characterized by an accumulation of extracellular matrix proteins. The initiation of fibrosis crucially depends on an inflammatory phase in which liver resident macrophages, Kupffer cells, are activated and release transforming growth factor β (TGF-β) as well as other proinflammatory cytokines that activate hepatic stellate cells (HSCs).[1,2] It is well established that Kupffer cells can originate from bone marrow mononuclear cells.[3] However, only recently, it has been appreciated that even in steady state

Abbreviations: ALT, alanine aminotransferase; CCR, C-C chemokine receptor; FACS, fluorescence activated cell sorting; HSC, hepatic stellate cell; Ig, immunoglobulin; iNOS, inducible nitric oxide synthase; MACS, magnetic-assisted cell sorting; MCP-1, monocyte chemoattractant protein 1; MIP-3α, macrophage inflammatory protein 3α; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; SD, standard deviation; α-SMA, α smooth muscle actin; T-bet, Th1-specific T box transcription factor; TGF, transforming growth factor; T_H, T helper; WT, wild-type.

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conditions Kupffer cells are constantly replenished by blood monocytes.[4] Although infiltration of blood-derived macrophages in addition to activation of classical Kupffer cells has been suggested as being essential for liver fibrogenesis,[5,6] the exact phenotype of infiltrating monocyte populations and the molecular mechanisms for hepatic recruitment are presently unknown.

Recently, two major monocyte subsets in mice have been identified that closely resemble human monocyte subsets and vary in migratory and differentiation properties.[7] In humans, classical $CD14^{++}CD16^{-}$ monocytes express C-C chemokine receptor (CCR) 2, CD64, and CD62L, whereas nonclassical $CD14^{+}CD16^{+}$ monocytes lack CCR2. Their counterparts in mice are $CCR2^{+}$ $Gr1^{hi}$ ($Ly6C^{hi}$) and $CCR2^{-}$ $Gr1^{lo}$ ($Ly6C^{lo}$) monocytes, respectively. $Gr1^{hi}$ monocytes are considered precursors for macrophages and dendritic cells in inflammatory conditions, whereas $Gr1^{lo}$ monocytes may represent steady state precursor cells for tissue macrophages.[8] The differential recruitment of these monocyte subsets appears to be crucially controlled by chemokines released from injured tissue. As such, enhanced hepatic expression of the ligands for CCR2, monocyte chemoattractant protein 1 (MCP-1)/CCL2, and CCR6, macrophage inflammatory protein 3α (MIP-3α)/CCL20, was described in patients with liver cirrhosis.[9,10] However, the specific roles of monocyte subsets in liver fibrosis are unknown.

In the present study, we examined the role of infiltrating monocyte subsets into the liver following acute and chronic injury and demonstrate an important functional role for inflammatory $Gr1^{hi}$ monocytes and their monocyte-derived intrahepatic macrophage subset for the progression of liver fibrosis.

Materials and Methods

Mice. C57BL/6, congenic Ly5.2 (CD45.1) C57BL/6, *Ccr2*$^{-/-}$, and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice (backcrossed to C57BL/6 background for more than eight generations) were maintained in our colony. All mice were housed in a pathogen-free environment. All experiments were performed with age- and sex-matched animals at 6 to 8 weeks of age under ethical conditions approved by the appropriate authorities according to German legal requirements.

Induction of Acute and Chronic Liver Injury or Liver Fibrosis. Mice received 0.6 mL/kg body weight of CCl_4 (Merck) mixed with corn oil intraperitoneally and were sacrificed at the indicated time points. For induction of liver fibrosis, CCl_4 was injected twice weekly for 6 weeks. Mice were sacrificed 48 hours after the last injection. As controls, animals received the same volume of the tracer (oil) intraperitoneally. Monocyte depletion was achieved by way of intravenous injection of 250 μL clodronate-loaded liposomes.[11]

Analysis of Blood and Intrahepatic Leukocytes. Whole blood was drawn and subjected to red cell lysis using Pharmlyse (BD), washed twice with Dulbecco's modified Eagle's medium containing 5 mM ethylene diamine tetraacetic acid and 0.5% bovine serum albumin and then stained with antibodies.[11] For flow cytometric analysis of intrahepatic leukocytes, livers were perfused with 20 to 40 mL phosphate-buffered saline (PBS), minced with scissors and subsequently digested for 30 minutes with collagenase type-IV (Worthington) at 37°C. Digested extracts were pressed through 70-μm cell strainers to gain single-cell suspensions. A small aliquot was stained with CD45 to assess the relative amount of intrahepatic leukocytes ($CD45^{+}$) among all liver cells. The remaining liver single cell suspension was subjected to density gradient centrifugation (LSM-1077, PAA) at 2,000 rpm for 20 minutes at 25°C. Leukocytes were collected from the interphase after centrifugation, washed twice with Hank's balanced salt solution containing 2% bovine serum albumin and 0.1 mM ethylene diamine tetraacetic acid, and subjected to fluorescence activated cell sorting (FACS).[12]

Flow Cytometry. Six-color staining was conducted using combinations of the following monoclonal antibodies: F4/80 (Serotec), CD115, CD4, CD11c, CD45.1, CD45.2, CD11b (all eBioscience), CD45, Gr1/Ly6C, Ly6G, CD19, NK1.1, CD8, CD3, mouse immunoglobulin (Ig) G1, rat IgG2a, or hamster IgG isotype controls (all BD). Flow cytometric analysis was performed on a FACS-Canto (BD) and analyzed with FlowJo (Tree Star).

Immunofluorescence. Liver sections were fixed in 4% paraformaldehyde, and immunohistochemical staining was performed according to standard procedures[11] using the following antibodies: anti-mouse CD11b (BD), F4/80 (Serotec), CD45.1 (BD), collagen I (Biotrend) or appropriate isotype controls and Cy3- or FITC-conjugated anti-IgG secondary antibodies (Jackson ImmunoResearch).

Histopathology and Alanine Aminotransferase. Conventional hematoxylin-eosin, Sirius red, and Ladewig stainings were performed according to standard protocols.[13] Alanine aminotransferase (ALT) activity (UV test at 37°C) was measured in serum (Roche Modular preanalytics system).

Real-Time Gene Expression Analysis. Livers were harvested and snap frozen in liquid nitrogen. RNA was purified from frozen liver samples by pegGOLD (peqLab), and complementary DNA was generated from 1 μg RNA using a complementary DNA synthesis kit

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(Roche). Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green Reagent (Invitrogen). Reactions were done twice in triplicate and β-actin values were used to normalize gene expression.[14] Primer sequences are available upon request.

Hydroxyproline Assay. Hepatic collagen was measured as described.[15] Briefly, liver samples were hydrolyzed with 6 N HCl at 110°C for 16 hours, then filtered and mixed with methanol and evaporated by a vacuum concentrator (Eppendorf). The crystallized samples were dissolved in 50% isopropanol and incubated with 0.6% chloramine-T for 10 minutes. One hundred microliters of freshly prepared Ehrlich's reagent was added, and samples were incubated at 50°C for 45 minutes under constant shaking. Samples were measured at 570 nm, and concentrations of total liver hydroxyproline were calculated against a standard curve.

Western Blotting. We performed sodium dodecyl sulfate electrophoresis of protein extracts and subsequent blotting as described,[16] using anti–α-SMA (Sigma) and glyceraldehyde 3-phosphate dehydrogenase (Biogenesis) antibodies.

Cytokine and Chemokine Measurements. TGF-β1 concentrations were measured from the liver extracts by way of enzyme-linked immunosorbent assay (BD). MCP-1 serum concentrations were measured using Cytometric Bead Array (BD).

Intracellular Staining of Cytokine Expression. Liver infiltrating leukocytes as described above were either solely cultured for inducible nitric oxide synthase (iNOS)/arginase-1 or stimulated with 100 μg/mL PMA and 10 μg/mL ionomycin (Sigma) for 4 hours at 37°C in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum. Cytokine secretion was blocked by adding 10 μg/mL Brefeldin-A (Sigma) for 2 hours at 37°C. Cells were stained with antibodies for surface markers (CD45, CD4, CD11b, F4/80) for 20 minutes and fixed with 2% formalin. For intracellular staining, fixed cells were permeabilized with BD Perm/Wash and incubated with antibodies against interleukin-4, interferon-γ, iNOS, arginase-1, or respective isotype controls (BD).

Adoptive Transfer of Monocytes. CD45.1$^+$ C57BL/6 mice were used as donors for monocyte transfer experiments. Monocytes were isolated from bone marrow of the femur and tibia by way of magnetic-assisted cell sorting (MACS) immunomagnetic cell selection using biotinylated CD115 monoclonal antibodies (eBioscience) followed by Streptavidin microbeads (Miltenyi).[17] Ninety to ninety-eight percent purity of isolated monocytes was confirmed by flow cytometry (as exemplified illustrated in Fig. 7B). Congenic CD45.2$^+$ C57BL/6 or *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ recipient mice were injected intravenously with 1 to 2 $\times$ 10^6 monocytes or PBS.

Coculture Experiments. The continuous cell line GRX having myofibroblastic characteristics[18,19] was obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, Brazil) and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 4 mM L-glutamine, and penicillin/streptomycin. For coculture experiments, GRX cells were platted into 6-well dishes and cultured for 24 hours. The medium was then renewed and the cells were either (1) left untreated; (2) stimulated with 5 ng/mL recombinant human TGF-β1 (R&D Systems); (3) cocultured with Gr1^{hi} bone marrow monocytes in the presence or absence of a polyclonal antibody against human TGF-β1 (sc-146; Santa Cruz Biotech); (4) cocultured with CD11b$^+$ F4/80$^+$Gr1$^+$ liver monocytes with or without the TGF-β1–specific antibody; or (5) cocultured with splenic CD19$^+$/B220$^+$ B cells or CD8$^+$ cytotoxic T cells. Murine immune cells were isolated from wild-type (WT) mice 48 hours after single CCl_4 intraperitoneal injection by MACS as described above. A purity >90% was ensured by FACS analysis. The cells were cocultured for 48 hours, and RNA was isolated for quantitative PCR (qPCR) as described above.

Statistical Analysis. All data are expressed as the mean + standard deviation (SD). Differences between groups were assessed by means of a two-tailed unpaired Student *t* test (SPSS).

Results

Gr1^{hi} but Not Gr1^{lo} Monocytes Are Massively Recruited into Injured Liver. To elucidate the role of monocyte subset infiltration following liver injury, C57BL/6 wild-type (WT) mice were challenged with carbon tetrachloride (CCl_4) intraperitoneally to induce an acute toxic hepatic damage. CCl_4 resulted in periportal necroses with maximal damage at 24 and 48 horus (Fig. 1A), also reflected by highly elevated serum ALT activities (Fig. 1B). Toxic damage was accompanied by a considerable influx of leukocytes into the liver (Fig. 1C), as assessed by way of FACS analysis. Hepatic leukocytes appeared predominantly mononuclear in hematoxylin-eosin histology (Fig. 1A). Infiltrating cells at early time points, especially at 48 hours after CCl_4 challenge, stained largely positive for the myeloid marker CD11b (Fig. 1D).

We further characterized the population of intrahepatic leukocytes, defined as CD45$^+$ cells, by way of FACS analysis. Using the myeloid marker CD11b and the macrophage marker F4/80 antigen, two distinct subsets of intrahepatic monocytes/macrophages could be identified:

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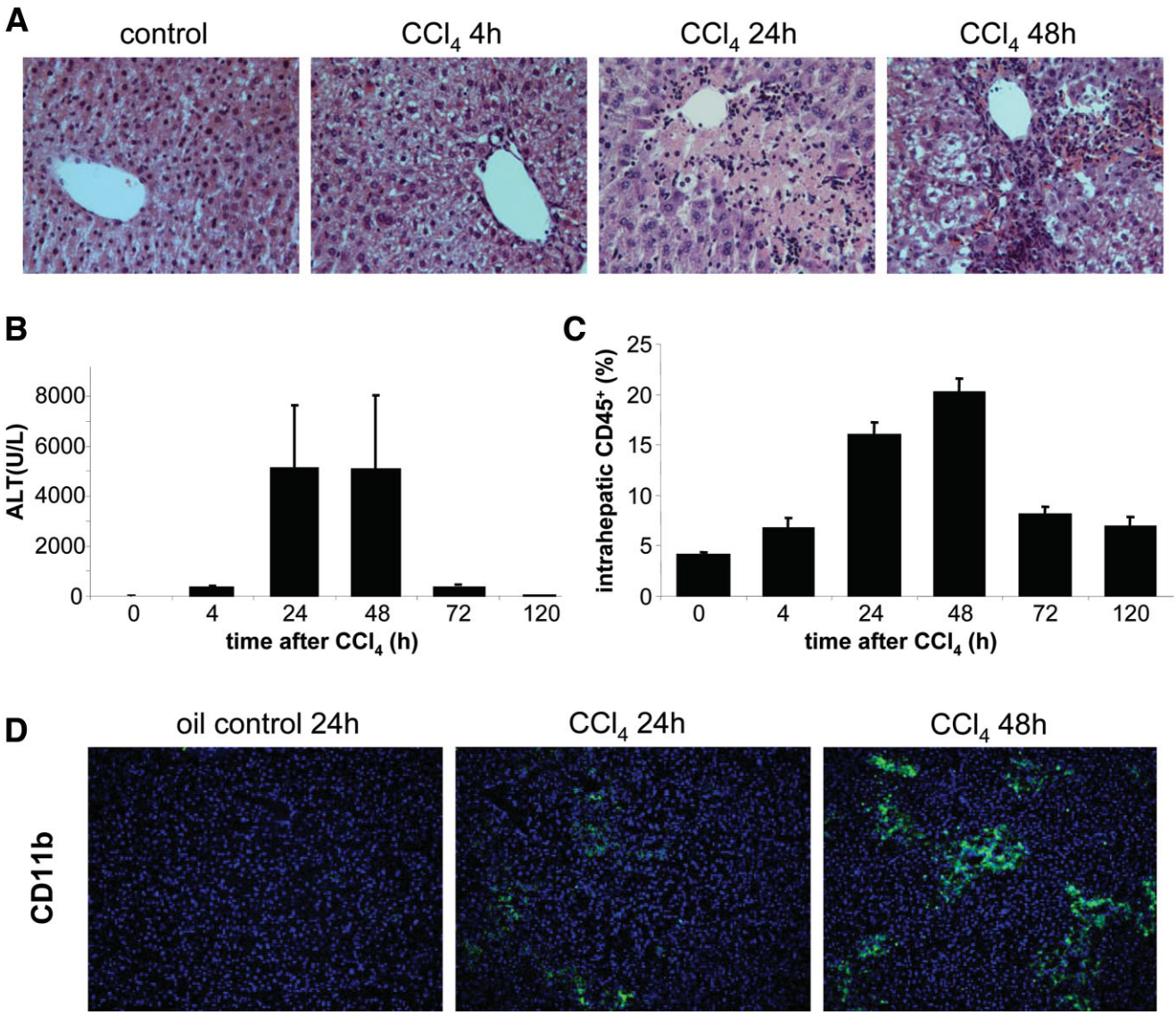

Fig. 1. Monocyte infiltration is a hallmark feature following acute liver injury. C57BL/6 WT mice were injected intraperitoneally with CCl_4 (0.6 mL/kg body weight) and analyzed at the time points indicated. Data are expressed as the mean ± SD from three independent experiments (n = 3 animals each per group). (A) Hematoxylin-eosin staining of liver paraffin sections shows progressive mononuclear infiltrates in the periportal regions after acute injury. (B) Time course of hepatic injury as indicated by serum ALT activity. (C) Infiltration of intrahepatic leukocytes (depicted as percentage of $CD45^+$ cells) parallels liver injury with a maximum at 48 hours after CCl_4 injection. (D) The periportal leukocyte infiltrates after acute liver injury largely stain positive for CD11b (green). DAPI nucleic counterstain appears in blue.

$CD11b^+F4/80^-$ and $CD11b^+F4/80^+$ cells (Fig. 2A). The $CD11b^+F4/80^+$ cells were found to express Gr1 (Ly6C) and variable levels of the dendritic cell marker CD11c (Fig. 2B), thereby resembling the phenotype of the peripheral $Gr1^{hi}$ monocyte subpopulation.[8] On the other hand, intrahepatic $CD11b^+F4/80^-$ cells expressed very low levels of Gr1 but stained positive for CD11c, corresponding to peripheral $Gr1^{lo}$ monocytes, which consistently also express the dendritic cell marker CD11c.[8] Both intrahepatic subsets differed from peripheral monocytes as they lacked expression of CD115 (M-CSF-receptor) that is expressed by blood monocytes and regularly down-regulated during monocyte differentiation into tissue macrophages or dendritic cells.[7] Both intrahepatic monocyte-derived subsets stained negative or minimal for Ly6G (neutrophil marker), CD3 (T cells), NK1.1 (natural killer cells), and CD19 (B cells) (data not shown).

Interestingly, the infiltration of the two subsets was differentially regulated following acute liver injury. The $CD11b^+F4/80^+Gr1^+$ subset massively increased after CCl_4 challenge, representing up to 50% of intrahepatic leukocytes 24 to 48 hours after damage (Fig. 2C) and 10% to 12% of the total liver cells (Fig. 2D). $CD11b^+F4/80^-$ cells only mildly increased after CCl_4 injection,

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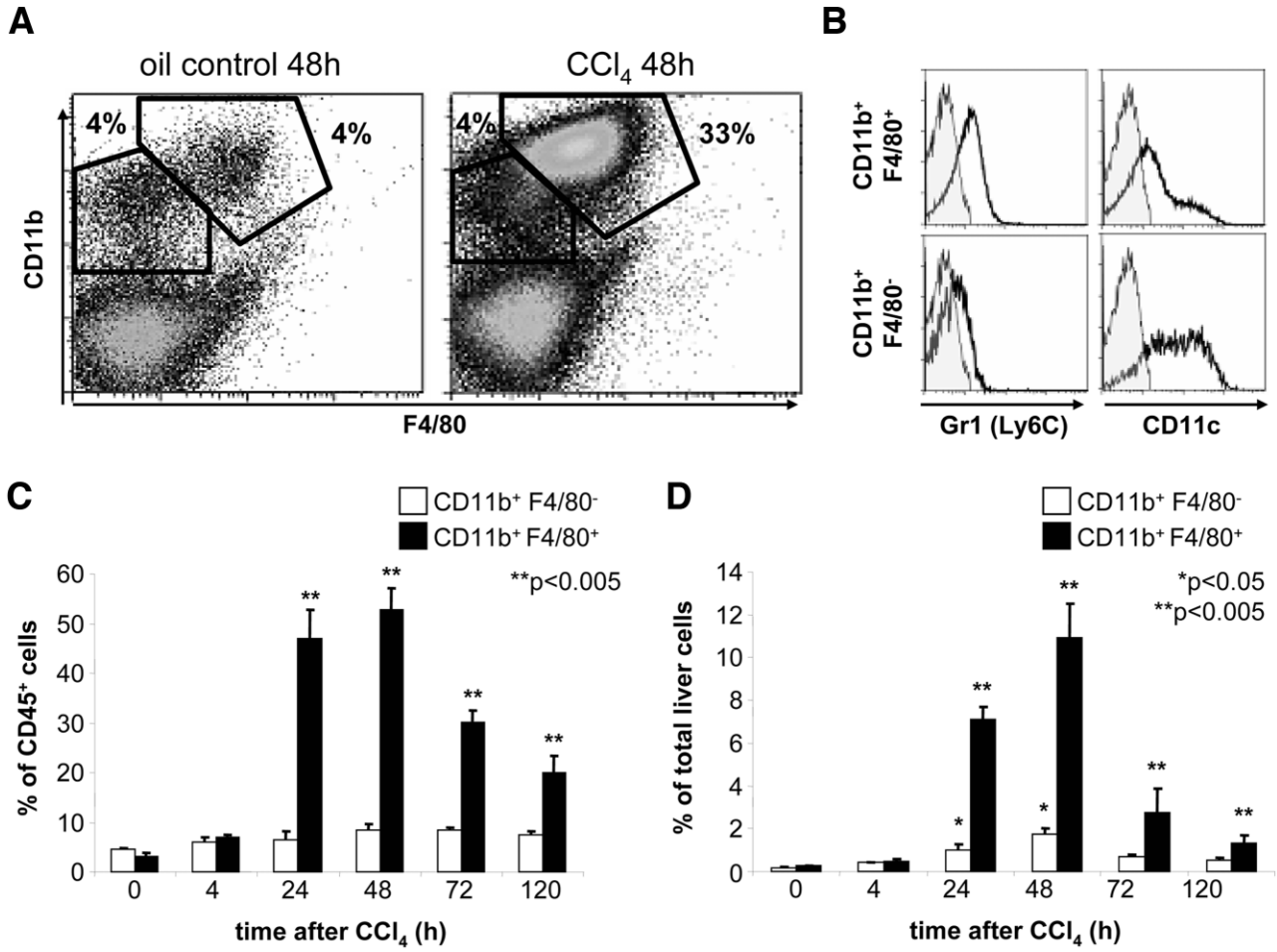

Fig. 2. Infiltrating hepatic monocytes consist of two different subsets with distinct kinetics after acute liver injury. C57BL/6 WT mice were injected intraperitoneally with CCl_4 (0.6 mL/kg body weight) and analyzed at the time points indicated. Data are expressed as the mean ± SD from three independent experiments (n = 3 animals each per group). (A) Flow cytometric analysis of intrahepatic leukocytes, gated on $CD45^+$ and live cells, reveals two subsets of CD11b and F4/80 expressing monocyte-derived cells. The $CD11b^+F4/80^+$ subset (right upper gate) tremendously increases after CCl_4-induced injury. (B) Flow cytometric analysis of intrahepatic monocyte-derived cells based on staining for CD45, CD11b, and F4/80. The $CD11b^+F4/80^+$ cells [right upper gate in (A)] express Gr1 (Ly6C) and variable levels of CD11c, the $CD11b^+F4/80^-$ population expresses low Gr1 and is positive for CD11c. Isotype controls are indicated in gray. (C) The relative contribution of the two monocyte-derived populations to the intrahepatic leukocytes ($CD45^+$ cells) after CCl_4 injury is depicted. (D) The relative contribution of the two monocyte-derived populations to total liver cells after CCl_4 is depicted. $*P < 0.05$ and $**P < 0.005$ versus baseline.

and their relative contribution to intrahepatic leukocytes remained constant (Fig. 2C-D).

To determine whether these monocyte-derived subsets actively endorse liver damage or rather represent an elemental reaction following the toxic injury, WT mice were treated with clodronate-loaded liposomes, which transiently deplete blood monocytes and liver macrophages.[11] During systemic monocyte/macrophage depletion, 12 hours after liposome injection, liver injury was induced by CCl_4 (Fig. 3A). Although monocytes were fully depleted from the blood (Fig. 3B,C) and liver (Fig. 3D), the magnitude of liver injury after CCl_4 was not affected (Fig. 3E), indicating that the differential recruitment of monocyte subsets into the injured liver does not directly promote the acute hepatic damage in this model.

CCR2 Is Critical for Recruitment of Gr1$^+$ Intrahepatic Monocyte-Derived Cells. The strong infiltration of $Gr1^{hi}$ monocytes after liver injury prompted us to investigate which chemokines might be involved in hepatic $Gr1^{hi}$ monocyte subset recruitment. $Gr1^{hi}$ monocytes express high levels of the chemokine receptor CCR2, and additionally CCR1 and CCR6, whereas $Gr1^{lo}$ monocytes express high levels of CX_3CR1, CCR5, and CCR6.[8] After acute CCl_4-induced liver injury, intrahepatic expression of the ligands for CCR2 and CCR6, MCP-1, and MIP-3α, respectively, was strongly up-regulated, whereas expression of fracta-

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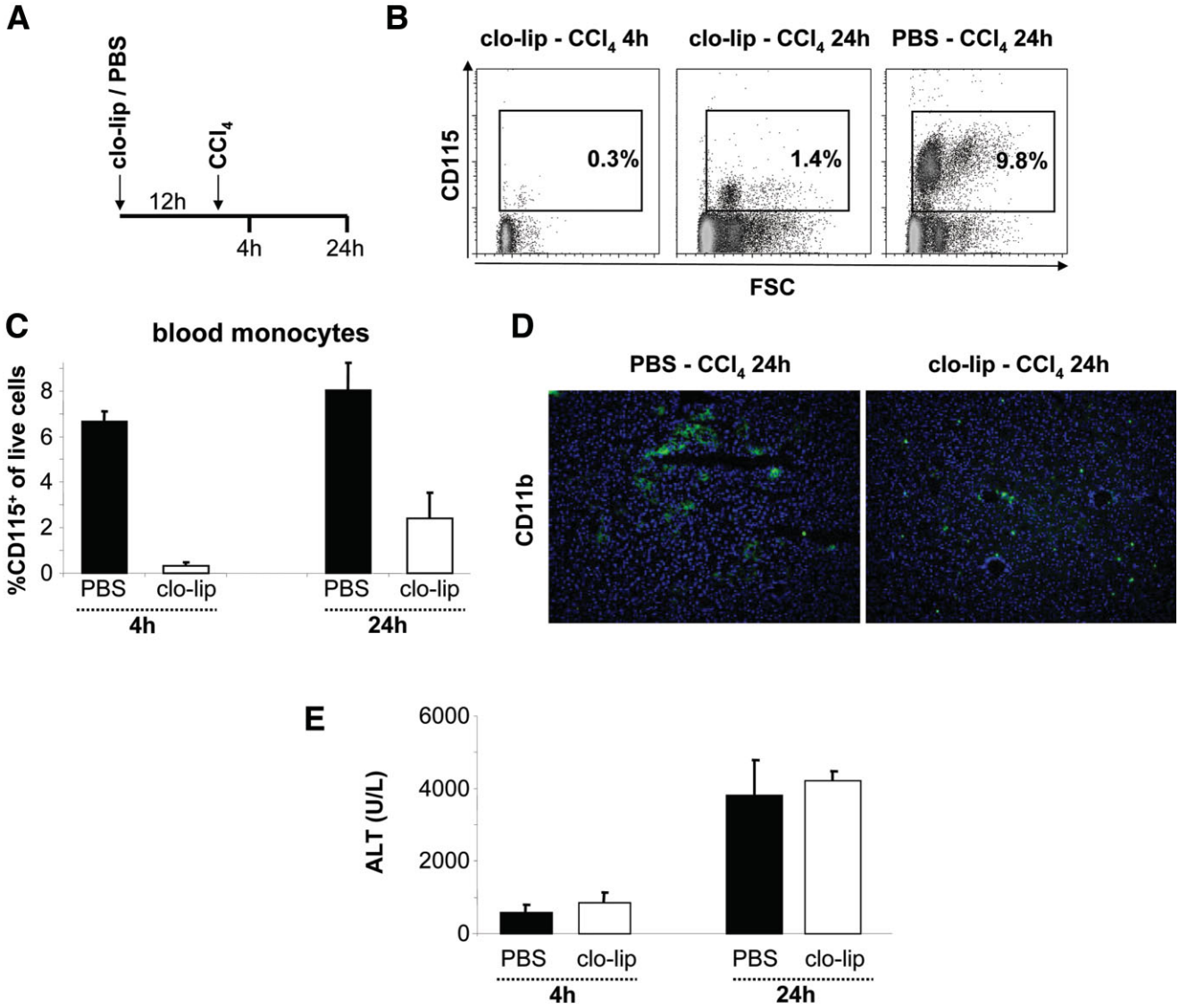

Fig. 3. Monocyte infiltration is dispensable for initiation of acute toxic liver damage. (A) Experimental design: C57BL/6 WT mice were either injected intravenously with clodronate-loaded liposomes (clo-lip) for monocyte depletion or with PBS-loaded control liposomes (PBS). During monocyte depletion, 12 hours after clo-lip, mice were injected intraperitoneally with CCl_4 (0.6 mL/kg body weight) and analyzed at the time points indicated. Data are expressed as the mean ± SD from three independent experiments (n = 3 animals each per group). (B) Representative flow cytometric analysis of peripheral blood monocytes (CD115). At 4 hours after CCl_4 (16 hours after clo-lip), peripheral blood monocytes are fully depleted (>90%). At 24 hours after CCl_4 (36 hours after clo-lip), some immature monocytes have returned to the circulation, but this is significantly less than for mice treated with PBS-loaded control liposomes. (C) Monocyte counts in peripheral blood after depletion and CCl_4 injection are depicted. (D) Administration of clo-lip also largely depletes resident and infiltrating monocytes/macrophages in the liver (right), as shown by immunohistochemistry for CD11b (green) in comparison with nondepleted CCl_4-injected controls (left). DAPI nucleic counterstain appears in blue. (E) Despite depletion of peripheral and intrahepatic monocytes/macrophages, hepatic injury after CCl_4 as quantified by serum ALT activity is similar in both groups.

lkine (CX_3CL1), the ligand for CX_3CR1, was only mildly induced (Fig. 4A).

When *Ccr2*- and *Ccr2/Ccr6*-deficient mice were challenged with CCl_4, the degree of hepatic damage was equal to WT mice (Fig. 4B), but leukocyte infiltration was significantly reduced after injury (Fig. 4C). The reduced leukocyte infiltration could be attributed to a significantly lower accumulation of $CD11b^+F4/80^+Gr1^+$ intrahepatic monocyte-derived cells in $Ccr2^{-/-}$ and $Ccr2^{-/-}$ $Ccr6^{-/-}$ mice (Fig. 4D). In contrast, the hepatic $CD11b^+F4/80^-$ cell population, corresponding to the $Gr1^{lo}$ monocytes, did not differ between WT, $Ccr2^{-/-}$, and $Ccr2^{-/-}Ccr6^{-/-}$ mice after CCl_4 injury (Supporting Fig. 1).

It was suggested previously that CCR2 might be primarily used by $Gr1^{hi}$ monocytes to exit the bone marrow, thereby regulating their accumulation in injured tissue.[20,21] After CCl_4-induced liver injury, se-

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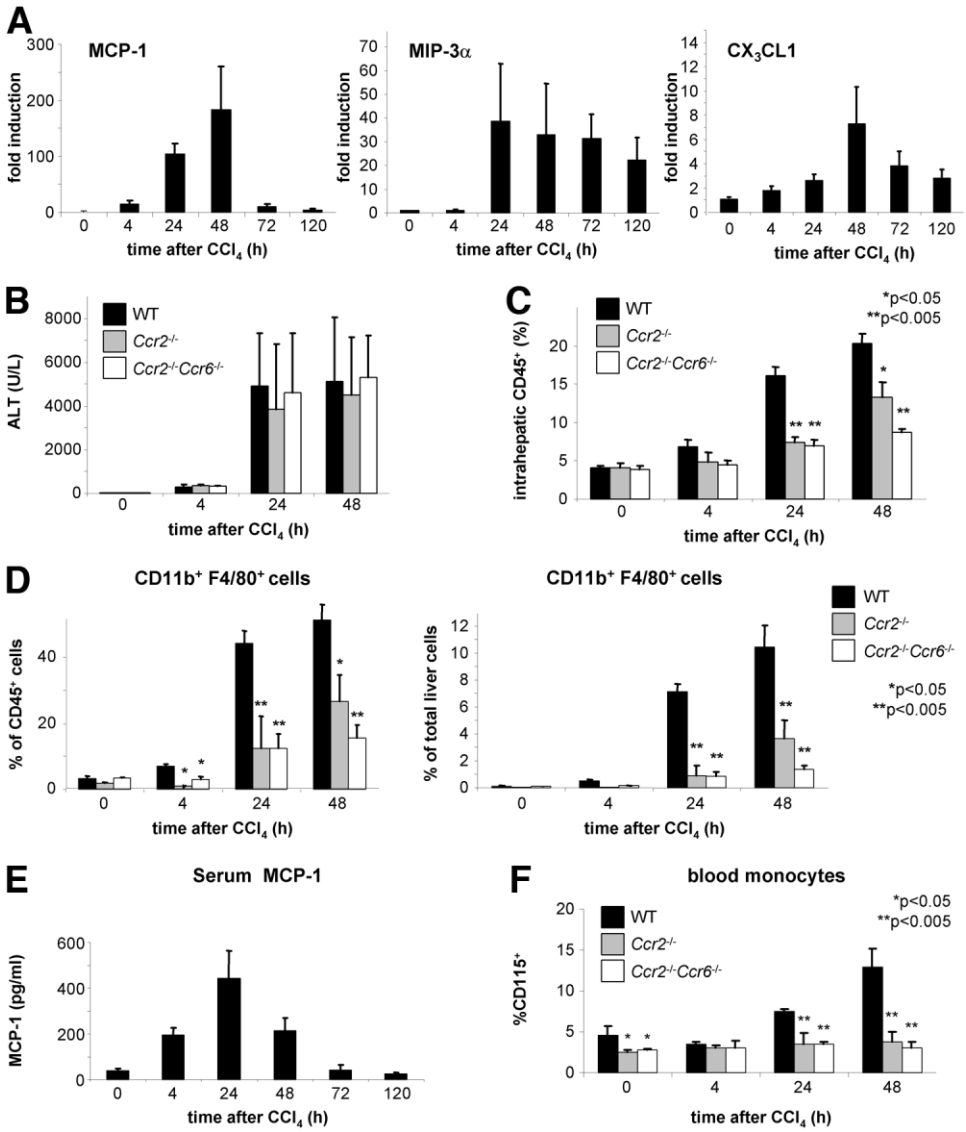

Fig. 4. Hepatic Gr1$^+$ monocyte subset recruitment is dependent on CCR2. C57BL/6 WT (black bars), *Ccr2*$^{-/-}$ (gray bars) and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ (white bars) mice were injected intraperitoneally with CCl_4 (0.6 mL/kg body weight) and analyzed at the time points indicated. Data are expressed as the mean ± SD from three independent experiments (n = 3 animals each per group). (A) Gene expression of MCP-1 (left), MIP-3α (middle), and CX_3CL1 (right) in liver from WT mice after CCl_4 injection. Results from qPCR are expressed as fold induction to baseline. (B) Time course of hepatic injury as indicated by serum ALT activity. (C) Infiltration of intrahepatic leukocytes (depicted as the percentage of CD45$^+$ cells) after CCl_4 administration. (D) The relative contribution of the CD11b$^+$F4/80$^+$ monocyte-derived cells to the intrahepatic leukocytes (CD45$^+$ cells, left graph) and to total liver cells (right graph) after injury by CCl_4 is depicted for WT, *Ccr2*$^{-/-}$, and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice. *$P < 0.05$ and **$P < 0.005$ versus WT. (E) Serum levels of MCP-1 increase in WT mice after CCl_4 injection and peak at 24 hours. (F) Blood monocyte counts increase in response to CCl_4 injection at 24 and 48 hours in WT, but not in *Ccr2*$^{-/-}$ or *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice. *$P < 0.05$ and **$P < 0.005$ versus WT.

rum MCP-1 was strongly up-regulated (Fig. 4E), in line with high hepatic MCP-1 expression after damage. Elevated serum MCP-1 preceded monocytosis in peripheral blood of WT mice (Fig. 4F). In contrast, *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice did not mobilize bone marrow monocytes into peripheral blood (Fig. 4F), although serum MCP-1 and bone marrow monocyte precursors were unaltered (data not shown). Thus, CCR2 is a critical factor for intrahepatic recruitment of CD11b$^+$F4/80$^+$Gr1$^+$ cells by mobilizing Gr1^{hi} monocytes from the bone marrow.

Intrahepatic Gr1$^+$ Monocyte-Derived Cells Are Also Increased During Liver Fibrogenesis, and Their Recruitment Is CCR2-Dependent. Although the acute damage is apparently not mediated by massive infiltration of Gr1^{hi} monocytes into the liver, Gr1^{hi} monocytes might critically regulate the intrahepatic processes occurring in response to injury. We therefore examined their role in a

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liver fibrosis model. Chronic administration of CCl_4 twice weekly for 6 weeks resulted in significant collagen deposition and liver fibrosis induction in WT mice concomitant with a pronounced monocytic infiltrate (Fig. 5A). Similar to the observations after acute injury, two subsets of monocyte-derived intrahepatic cells could be distinguished in the fibrotic liver (Fig. 5B). However, only the $CD11b^+F4/80^+Gr1^+$ subset was largely increased during fibrogenesis (Fig. 5C), while the $CD11b^+F4/80^{low}$ subset remained unaltered (not shown). Both intrahepatic subsets originated primarily from infiltrating monocytes and not from proliferating resident macrophages, as they stained mostly negative for the proliferation marker Ki-67 during different stages of chronic liver injury (data not shown).

Ccr2$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice displayed a significant reduction in the intrahepatic $CD11b^+F4/80^+Gr1^+$ population in comparison to WT mice also in the chronic injury model (Fig. 5C). Decreased accumulation of intrahepatic $CD11b^+F4/80^+Gr1^+$ cells was associated with a diminished development of liver fibrosis, as evidenced by reduced collagen staining (Fig. 5D), hepatic hydroxyproline content (Fig. 5E), and HSC activation (α-SMA expression) (Fig. 5F). These data demonstrate a critical role for CCR2 in the recruitment of $Gr1^{hi}$ monocytes upon chronic liver injury, which appear to bear a crucial function in the development of liver fibrosis.

Hepatic Gr1$^+$ Monocyte-Derived Cells Preferentially Constitute Proinflammatory iNOS-Producing Macrophages and Direct T Cell Differentiation During Liver Fibrogenesis. We further investigated the mechanisms by which infiltrating $Gr1^{hi}$ monocytes promote hepatic fibrosis. Macrophages are known to be the primary source of TGF-β1 in the fibrotic liver, which is regarded as the major profibrogenic cytokine for the activation of HSCs.[1] Consistent with the reduced accumulation of intrahepatic $CD11b^+F4/80^+Gr1^+$ monocyte-derived cells and diminished fibrogenesis in *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice, *Tgfb1* gene expression and TGF-β1 protein levels were markedly lower in knockout compared with WT animals after 6 weeks of CCl_4 (Fig. 6A).

Macrophages can principally differentiate into at least two functionally distinct populations during chronic inflammatory and fibrotic processes: 'classically activated' M1-type macrophages express iNOS and favor a T helper (T_H) 1–T cell environment, 'alternatively activated' M2-type macrophages express arginase-1 and preferentially interact with T_H2 T-cells.[22] After 6 weeks of CCl_4 treatment, hepatic gene expression of arginase-1 was higher and iNOS was lower in *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice compared to WT mice that showed up-regulation of both factors in response to CCl_4-induced damage (Fig. 6B). On FACS analysis, more intrahepatic $CD11b^+F4/80^+$ cells differentiated into iNOS- than into arginase-1-expressing macrophages in WT as compared to *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice (Fig. 6C+D), suggesting a preferential differentiation of $Gr1^{hi}$ monocytes into iNOS-producing $CD11b^+F4/80^+$ macrophages during liver fibrogenesis. Consistently, *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice had lower hepatic expression of Th1-specific T box transcription factor (T-bet) as a marker of T_H1 differentiation and markedly reduced levels of interferon-γ–producing T_H1 cells in the fibrotic liver than WT animals, whereas the T_H2 differentiation was not altered (Fig. 6E,F). These data show that infiltrating $Gr1^{hi}$ monocytes mainly constitute classically activated iNOS-producing macrophages in the liver and contribute to establishing the hepatic T_H1 T cell response during fibrogenesis.

The Infiltrating Gr1$^+$ Monocyte Subpopulation Promotes Profibrogenic Actions at Different Stages of Liver Fibrosis. To further specify the intrahepatic differentiation potential and functional contribution of $Gr1^{hi}$ monocytes to liver fibrosis *in vivo*, we adoptively transferred purified $CD45.1^+$ $Gr1^{hi}$ monocytes into $CD45.2^+$ WT or *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ recipient mice at different time points of CCl_4-induced chronic liver damage (Fig. 7A,B). Adoptively transferred $Gr1^{hi}$ monocytes trafficked into the liver and could be found in the periportal regions of the fibrotic liver (Supporting Fig. 2A). Even as late as 3 to 6 weeks after adoptive transfer, about half of the $Gr1^{hi}$-monocyte-derived cells were still $CD11b^+F4/80^+$ macrophages, whereas the other cells had a more differentiated $CD11b^-F4/80^+$ resident macrophage phenotype (Supporting Fig. 2B).

$Gr1^{hi}$ monocytes were either transferred within the first (early transfer) or final (late transfer) 3 weeks of the 6-week CCl_4 treatment (Fig. 7A). In both conditions, $Gr1^{hi}$ monocytes strongly enhanced progression of liver fibrosis (Fig. 7C,D). Interestingly, the transfer of WT $Gr1^{hi}$ monocytes not only restored fibrogenesis in *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice to WT levels, but even increased HSC activation and collagen deposition in both WT and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice after $Gr1^{hi}$ monocyte transfer (Fig. 7C-D). Consequently, collagen deposition was higher after early transfer than in mice that had received monocytes at later periods of CCl_4 treatment, due to the prolonged enhanced HSC activation (Fig. 7E-F).

Intrahepatic Gr1$^+$ Monocytes Directly Activate HSCs Through TGF-β. The adoptive transfer of purified $Gr1^{hi}$ monocytes demonstrated the efficient profibrogenic potential of bone marrow–derived monocyte subsets in experimental liver fibrosis. However, it was not clear if this is a general feature of $Gr1^{hi}$ blood/bone marrow monocytes per se or if prior differentiation in the inflamed liver microenvironment is required. Furthermore, we wanted to ensure that this is a monocyte subset-specific effect and aimed at unrav-

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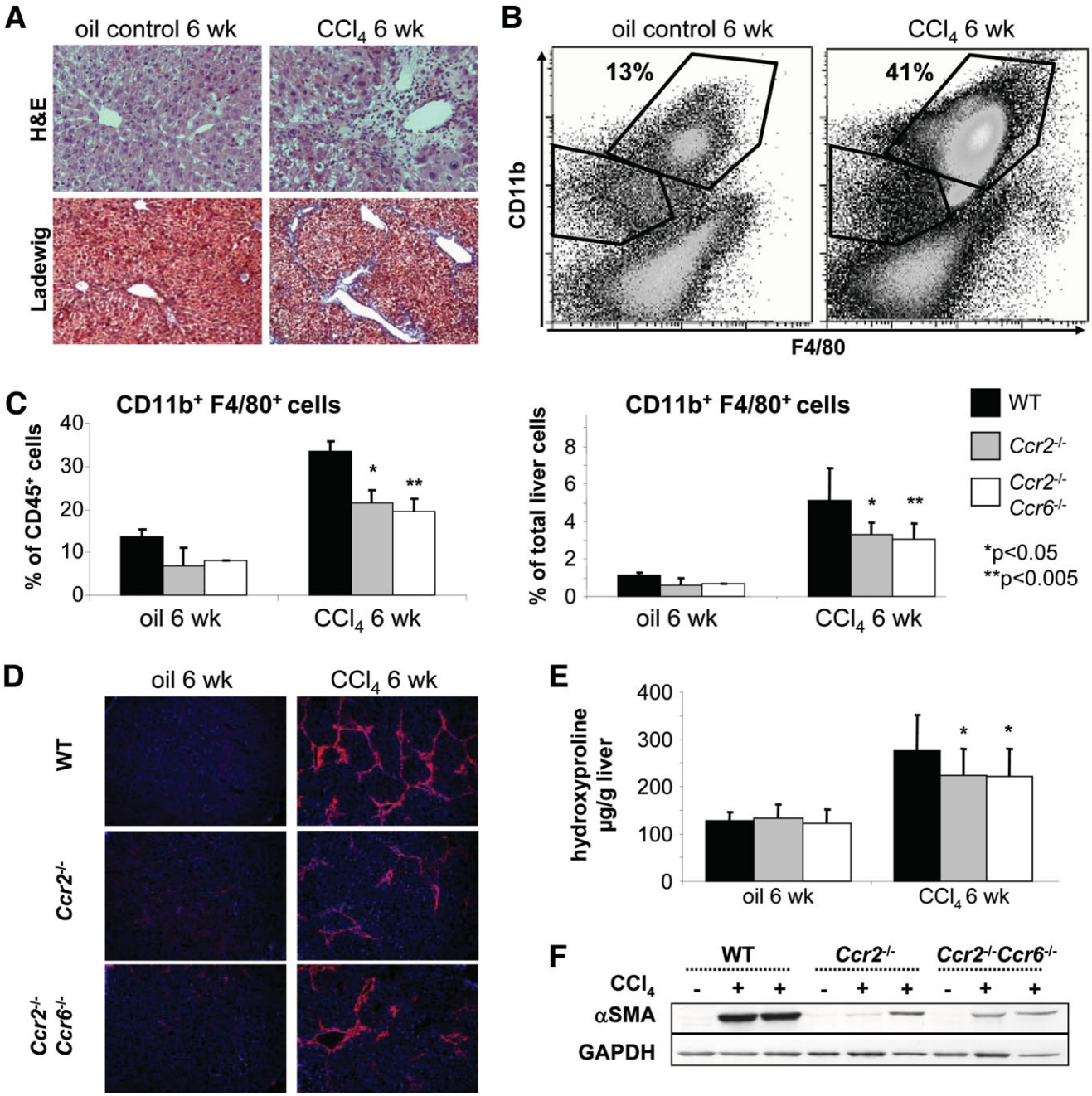

Fig. 5. Reduced Gr1$^+$ monocyte subset recruitment during chronic liver injury is associated with reduced liver fibrogenesis. C57BL/6 WT (black bars), *Ccr2*$^{-/-}$ (gray bars), and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ (white bars) mice were injected intraperitoneally with CCl_4 (0.6 mL/kg body weight) or tracer (oil control) twice weekly for 6 weeks. Data are expressed as the mean ± SD from three independent experiments (n = 5 animals each per group). (A) Induction of liver fibrosis in WT animals after 6 weeks of CCl_4 injections is accompanied by persistent periportal infiltrates of mononuclear cells as shown in hematoxylin-eosin or Ladewig (blue: collagen fibers) staining. (B) Similar to acute CCl_4-mediated injury (compare to Fig. 2), a marked increase in CD11b$^+$F4/80$^+$ monocyte-derived cells (upper right gate) is evident by flow cytometric analysis of intrahepatic leukocytes (pregated on CD45$^+$ and live cells) of WT mice after 6 weeks of CCl_4 injections as compared with tracer (oil)-injected controls. (C) The relative contribution of the CD11b$^+$F4/80$^+$ monocyte-derived cells to the intrahepatic leukocytes (CD45$^+$ cells, left graph) and to total liver cells (right graph) after chronic injury by CCl_4 is depicted for WT, *Ccr2*$^{-/-}$, and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice. *$P < 0.05$ and **$P < 0.005$ versus WT. (D) The extent of liver fibrosis after 6 weeks of CCl_4 was assessed by immunohistochemistry for collagen (red) showing reduced collagen deposition in *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice. Representative individual mice are shown. (E) The hydroxyproline content of the livers after 6 weeks of CCl_4 is also reduced in *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice. *$P < 0.05$ versus WT. (F) Western blot analysis reveals reduced α-SMA after 6 weeks of CCl_4 in *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice. Protein extracts of individual mice are shown that were either treated with CCl_4 (+) or oil (−) intraperitoneally. Glyceraldehyde 3-phosphate dehydrogenase is used to ensure equal loading.

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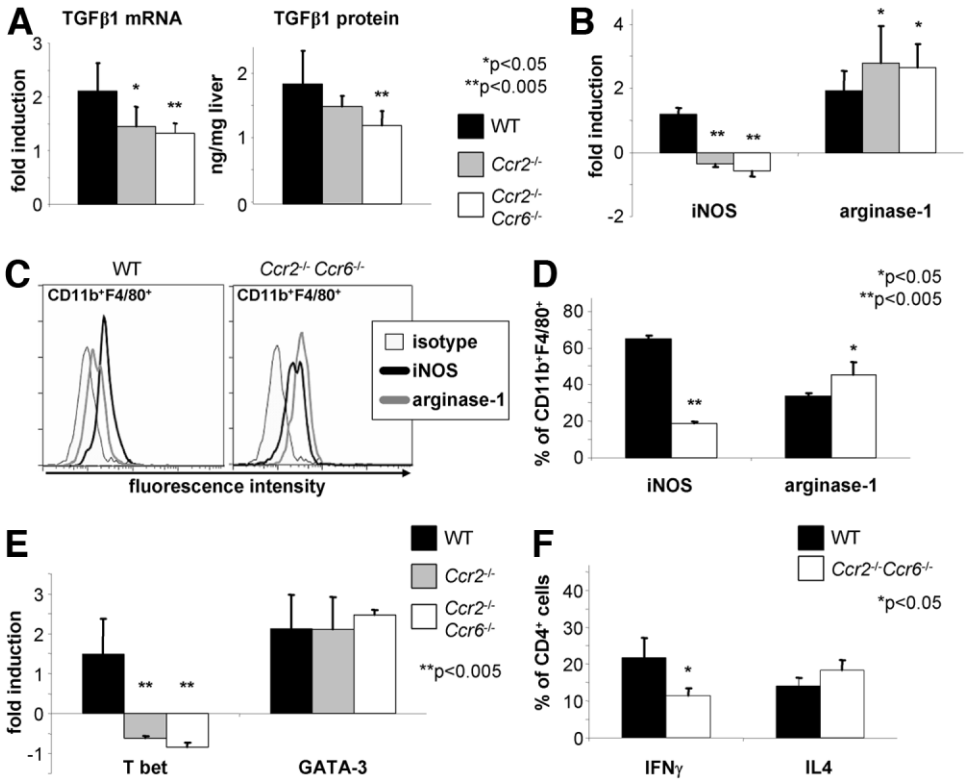

Fig. 6. $Gr1^+$ monocyte subset recruitment is required for inflammatory macrophage and T cell differentiation during liver fibrogenesis. C57BL/6 WT (black bars), *Ccr2*$^{-/-}$ (gray bars) and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ (white bars) mice were injected intraperitoneally with CCl_4 (0.6 mL/kg body weight) or tracer (oil control) twice weekly for 6 weeks. Data are expressed as the mean ± SD from three independent experiments (n = 3 animals each per group). (A) Hepatic TGF-β1 gene (qPCR, left) and protein expression (enzyme-linked immunosorbent assay, right) is reduced in knockout animals. Results from qPCR are expressed as fold induction to control (oil)-injected WT mice. (B) Hepatic gene expression for iNOS and arginase-1 by qPCR shows reduced iNOS and higher arginase-1 messenger RNA levels in *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice compared with WT. Results from qPCR are expressed as fold induction to control (oil)-injected WT mice. (C) iNOS and arginase-1 protein expression was assessed by intracellular staining of hepatic leukocytes after 6 weeks CCl_4. Representative histograms show iNOS (black line) and arginase-1 (dark gray line) expression in comparison to isotype control (light gray solid curve) gated on intrahepatic $CD45^+CD11b^+F4/80^+$ cells in WT (left) and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice (right). (D) Relative numbers of either iNOS- or arginase-1–expressing intrahepatic $CD11b^+F4/80^+Gr1^+$ cells (in percentage of all intrahepatic $CD11b^+F4/80^+$ cells) after 6 weeks CCl_4, based on FACS analysis. (E) Hepatic gene expression for T-bet (transcriptor factor of T_H1 cells) and GATA-3 (transcriptor factor of T_H2 cells) by qPCR shows reduced T-bet messenger RNA levels in *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice compared with WT. Results from qPCR are expressed as fold induction to control (oil)-injected WT mice. (F) Interferon-γ and interleukin-4 protein expression by intrahepatic $CD4^+$ T cells was assessed by intracellular staining of hepatic leukocytes after 6 weeks CCl_4.

eling the underlying molecular mechanisms of monocyte-mediated HSC activation. Therefore, the intrahepatic $CD11b^+F4/80^+Gr1^+$ monocyte subset was isolated from CCl_4-injected mice and tested in vitro in comparison with purified $Gr1^{hi}$ bone marrow monocytes as well as control B cells and $CD8^+$ T cells (Fig. 8A). Monocytes or control cells were cocultured with the murine GRX cell line that has typical characteristics of HSCs, including the potential to transform into myofibroblasts[18,19] (Fig. 8B). In fact, stimulation with recombinant TGF-β–induced expression of α-SMA and collagen in GRX cells within 48h (Fig. 8C). The coculture experiments revealed that only liver-derived $Gr1^+$ monocytes, but not bone marrow monocytes or B or $CD8^+$ T cells, were able to induce α-SMA and collagen expression in GRX cells (Fig. 8C). This HSC activation could be blocked by a TGF-β–specific antibody (Fig. 8C). This demonstrates that $Gr1^{hi}$ monocytes, after their recruitment into the liver, undergo activation and differentiation in the inflamed hepatic environment before exerting specific profibrogenic actions.

Discussion

Here, we provide evidence for a vital role of monocyte subset infiltration for the development of liver fibrosis upon chronic hepatic injury. We demonstrate that $Gr1^+$ ($Ly6C^+$) inflammatory monocytes are predominantly recruited after acute and during chronic liver injury and give rise to iNOS-producing $CD11b^+F4/80^+$ intrahepatic macrophages. The phenotype of the $CD11b^+F4/80^+$ monocyte-derived cells after liver injury has striking similarities to monocyte-derived cells in other organs

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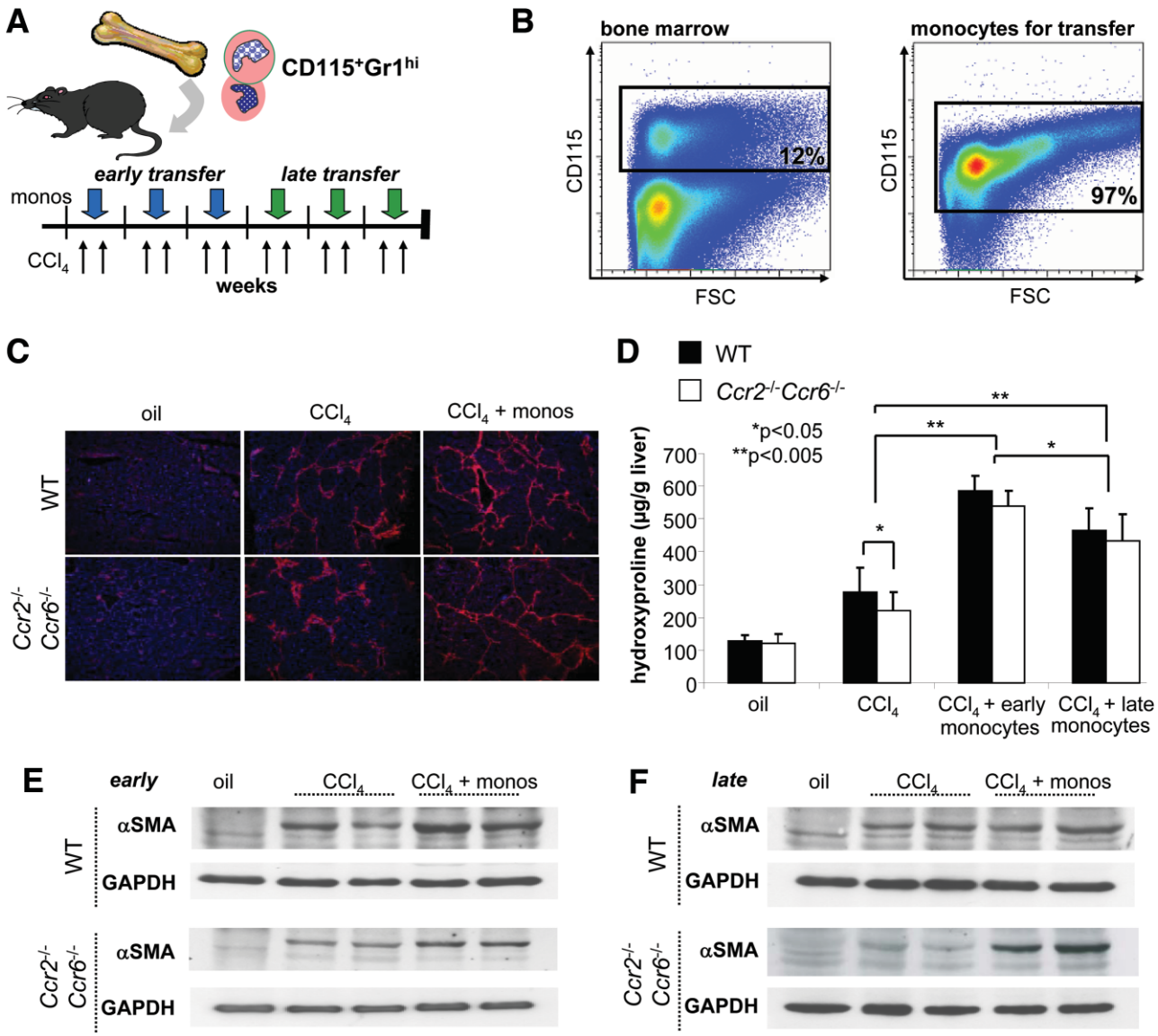

Fig. 7. Adoptive Gr1$^+$ monocyte transfer promotes liver fibrogenesis during early and late stages of chronic injury. (A) Experimental design: C57BL/6 WT or *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice (all CD45.2$^+$) were injected intraperitoneally with CCl_4 (0.6 mL/kg body weight) or tracer (oil control) twice weekly for 6 weeks. Gr1$^+$ monocytes were isolated from bone marrow of CD45.1$^+$ mice by MACS and injected intravenously into CCl_4-treated recipients either at weeks 1, 2, and 3 (early transfer) or at weeks 4, 5, and 6 (late transfer) of treatment, respectively. (B) Bone marrow from WT mice contained 7% to 15% monocytes identified by positive staining for CD115 (M-CSF receptor, left representative FACS plot). Bone marrow monocytes were isolated by MACS with a purity of >90% (right representative FACS plot: CD115$^+$ monocytes after MACS separation) and used for adoptive transfer experiments. (C) Representative immunohistochemistry of liver sections for collagen (red) shows enhanced fibrosis progression in mice that have received adoptively transferred monocytes. Individual mice from the early transfer regimen are depicted, 6 weeks after CCl_4 treatment. (D) The hydroxyproline content of the livers for WT (black bars) and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice (white bars) was assessed for the different experimental conditions, 6 weeks after CCl_4 treatment. Data are expressed as the mean ± SD from two independent experiments (n = 4 animals each per group). *$P < 0.05$ and **$P < 0.005$. Hydroxproline content did not differ between WT and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ in mice that have received adoptive monocyte transfers. (E, F) Western blot analysis reveals enhanced α-SMA after (E) early and (F) late monocyte transfer in WT and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice. Protein extracts of individual mice are shown 6 weeks after CCl_4 treatment.

after injury. It shares the expression of Gr1/Ly6C while lacking Ly6G or high amounts of CD11c with inflammatory monocytes that are recruited into the ileum and peritoneal cavity after infection with the parasite *Toxoplasma gondii*.[23] It also shares the preferential differentiation into iNOS-producing cells in the chronic injury model of *Listeria monocytogenes* infection, in which inflammatory monocytes are recruited into the spleen.[20,24]

Accumulation of Gr1^{hi} monocytes in the injured liver is critically dependent on the chemokine receptor CCR2. Increasing experimental evidence suggests that the chemokine receptor CCR2 regulates Gr1^{hi} monocyte entry into inflamed tissue, mainly indirectly, by promoting the egress of Gr1^{hi} monocytes from the bone marrow into the circulation.[20,21] Our data demonstrate that this mechanism also applies to liver injury, because toxic liver damage resulted in

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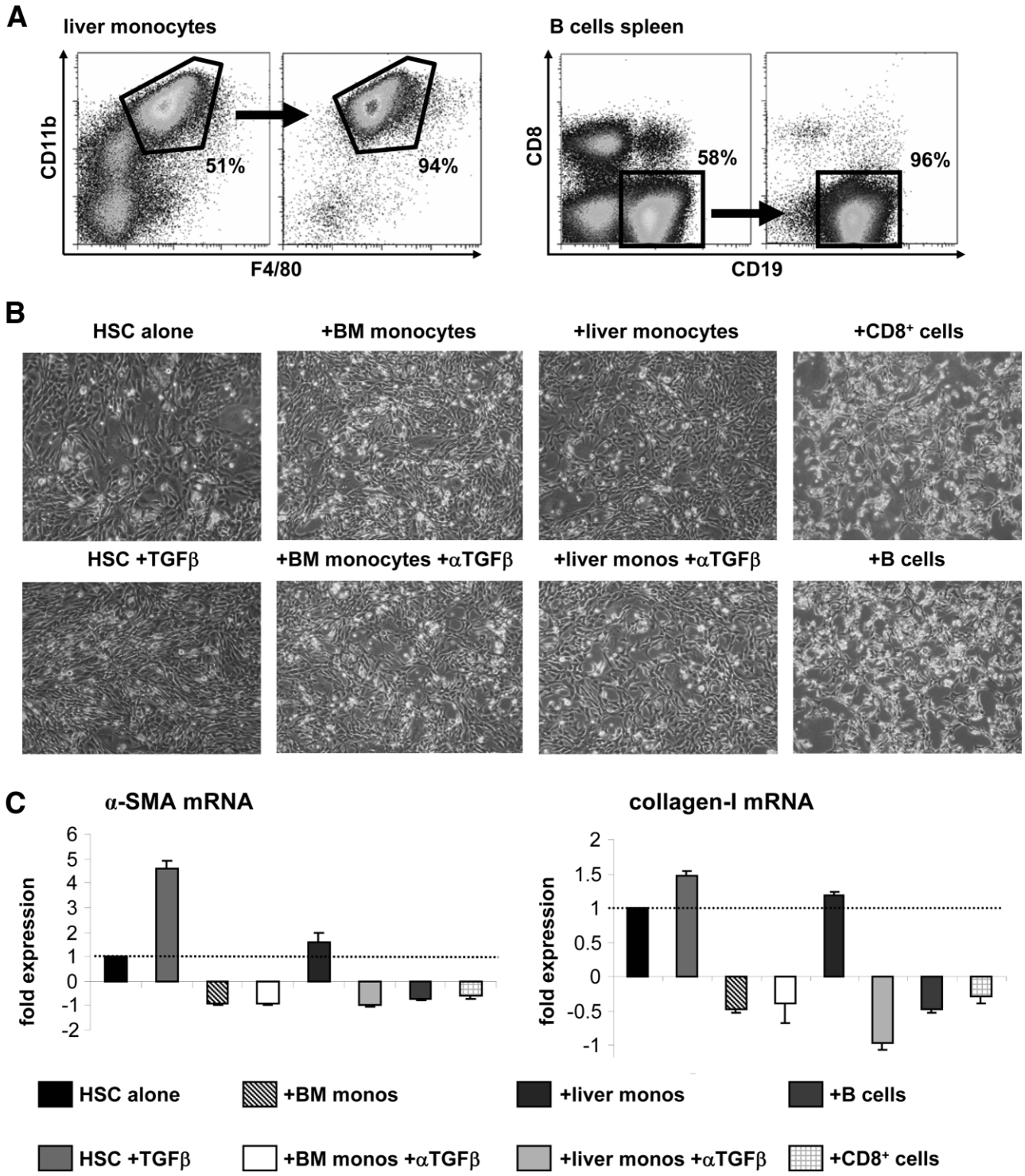

Fig. 8. Intrahepatic Gr1$^+$ monocytes directly promote HSC activation through TGF-β. (A) Forty-eight hours after intraperitoneal CCl_4, CD11b$^+$F4/80$^+$Gr1$^+$ monocytes were isolated from perfused livers by way of MACS, resulting in purities of 93% to 97%. Representative FACS analysis before (left) and after isolation (second graph from left) is shown. Bone marrow Gr1^{hi} monocytes were isolated by CD115 (compare to Fig. 7B). B cells were isolated by B220 microbeads from spleen by way of MACS; representative FACS analysis before (second from right) and after isolation (right, 96% CD19$^+$ B cells) is shown. (B) The HSC cell line GRX was cocultured with different immune cell subsets in the absence or presence of anti–TGF-β1 antibodies (αTGF-β) as indicated. As a positive control, GRX cells were stimulated with recombinant TGF-β1 (left lower corner). (C) Forty-eight hours after coculture, RNA was isolated, and the messenger RNA expression for α-SMA and type 1 collagen was quantified using real-time PCR. Results from qPCR are expressed as fold induction to HSCs alone. The coculture experiments were performed in triplicate.

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a sequence of increased hepatic MCP-1 expression, increased serum MCP-1, and peripheral blood monocytosis in WT animals. In contrast, *Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice lacked peripheral blood monocytosis after injury and subsequently harbored significantly less Gr1^{hi} monocyte-derived CD11b^{+}F4/80^{+} intrahepatic macrophages. Adoptively transferred WT Gr1^{hi} monocytes did traffic into the injured liver of *Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice, corroborating that CCR2 primarily promotes egress of Gr1^{hi} monocytes from the bone marrow and not immigration into the injured liver. CCR6, on the other hand, did not procure an additional effect on monocyte subset immigration, unlike in the inflamed skin,[25,26] although hepatic expression of MIP-3α, the CCR6 ligand, was up-regulated after injury. However, it is important to note that especially upon chronic injury, alternative pathways of monocyte subset recruitment can partially compensate for the function of CCR2/MCP-1. This has also been demonstrated in other chronic inflammatory models such as atherosclerosis, and CCR1, CCR5, and CX_3CR1 have been identified as chemokine receptors involved in Gr1^{hi} monocyte migration.[7,8,27] Their roles in liver fibrosis are currently unknown and require further study.

Several observations support our conclusion that Gr1^{hi} inflammatory monocytes are critical factors in liver fibrosis. First, the Gr1^{hi} monocyte-derived intrahepatic CD11b^{+}F4/80^{+} cells were up-regulated after acute injury by almost 10-fold and remained up to five-fold elevated during chronic liver damage. Second, in the absence of CCR2, CCl_4-treated animals displayed a significantly attenuated fibrosis progression. Third, adoptive transfer of purified Gr1^{hi} monocytes into CCl_4-treated animals dramatically increased HSC activation, collagen deposition, and fibrogenesis. In line, selective depletion of (total) monocytes/macrophages during fibrosis progression in CD11b-DTR mice has been found to reduce hepatic fibrosis.[5] Of note, the adoptive transfer of Gr1^{hi} monocytes even enhanced fibrosis in WT mice. In this set of experiments, purified monocytes were repeatedly injected 48 hours after CCl_4, when the peak of endogenous intrahepatic monocyte infiltration was observed, for several weeks. The additional profibrogenic effect of adoptively transferred monocytes suggests that the pathways preferentially recruiting Gr1^{hi} monocytes into the chronically injured liver are highly up-regulated and are not fully saturated by the endogenous monocyte influx.

A possible molecular mechanism wherein macrophages interact with HSC during liver fibrogenesis is the release of several factors, including TGF-β1, platelet-derived growth factor, or tumor necrosis factor α, that are important activators of HSCs.[1] HSCs, on the other hand, express MCP-1/CCL2 and also the chemokines CCL3, CCL4, CXCL1, CXCL2, or CXCL10 after activation via Toll-like receptor 4–dependent signals, thereby facilitating monocyte/macrophage chemotaxis during fibrogenesis.[28,29] Our *in vivo* data suggested that intrahepatic CD11b^{+}F4/80^{+} monocyte-derived cells, just like liver resident macrophages, produce TGF-β1 and thereby directly activate HSCs. This was confirmed in an *in vitro* experiment, in which only intrahepatic recruited monocytes could readily (within 48 hours of coculture) activate HSCs in a TGF-β–dependent manner.

Our experiments further indicate that macrophage differentiation has an impact on the local T cell environment in liver fibrogenesis. T_H2 cell–derived cytokines—namely interleukin-4, interleukin-5, and interleukin-13—can enhance fibrosis progression by stimulating TGF-β production in macrophages and by direct effects on myofibroblasts.[30] In our model, intrahepatic accumulation of Gr1^{hi} monocyte-derived cells was reduced in *Ccr2*$^{-/-}$ mice, accompanied by a lack of appropriate T_H1 responses in the chronically injured liver. Impaired T_H1-type cytokine responses in *Ccr2*$^{-/-}$ mice have been previously attributed to alterations of monocyte migration.[31] Our data now indicate that the magnitude of CD11b^{+}F4/80^{+} intrahepatic cells guides the local T_H1/T_H2 environment in liver fibrogenesis, preferentially governing T_H1-driven immune responses. However, further experiments are needed to clarify if the lack of T_H1 responses observed in our model functionally contributes to reduced fibrosis or if the lack of monocyte-mediated HSC activation by itself fully explains the phenotype.

In this study, we define intrahepatic monocyte-derived subsets in experimental murine liver injury and identify Gr1^{hi} monocytes as precursors of functionally important CD11b^{+}F4/80^{+} intrahepatic macrophages during acute and chronic hepatic damage. These findings are likely to be of key relevance for human liver disease. It has been reported that the number of macrophages increases during chronic liver injury and fibrogenesis.[1,2] Mouse Gr-1^{hi} monocytes are thought to resemble the human CD14^{++}CD16^{-}, and Gr-1^{lo} the human CD14^{+}CD16^{+} subset.[7] Human CD14^{++}CD16^{-} shares many characteristics with the murine Gr1^{hi} monocytes, including activation markers or CCR2 chemokine receptor expression. In patients with liver cirrhosis, both increased peripheral CD14^{++}CD16^{-} and CD14^{+}CD16^{+} monocytes have been reported in the literature.[32,33] Moreover, consistent with our findings in murine liver injury, up-regulated intrahepatic and systemic levels of MCP-1/CCL2 as well as MIP-3α/CCL20 were described in patients with liver cirrhosis.[9,10,34,35] We confirmed the up-regulation of MCP-1/CCL2, but also CCR2, suggesting accumulation of CCR2^{+} monocytes, in liver biopsies from patients with cirrhosis compared with normal liver (unpublished observations). Further investigations are needed to

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translate the findings from the animal models into human pathogenesis. Nevertheless, our experiments from murine liver injury suggest that the modulation of monocyte subset recruitment into the liver and subsequent differentiation in the inflamed hepatic environment may represent possible novel approaches for interventions targeting proinflammatory and profibrogenic actions of $Gr1^{hi}$ monocytes (or their human counterparts) in chronic liver diseases and liver fibrosis.

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References

1. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115:209-218.
2. Karlmark KR, Wasmuth HE, Trautwein C, Tacke F. Chemokine-directed immune cell infiltration in acute and chronic liver disease. Expert Rev Gastroenterol Hepatol 2008;2:233-242.
3. Gale RP, Sparkes RS, Golde DW. Bone-marrow origin of hepatic macrophages (Kupffer cells) in humans. Science 1978;201:937-938.
4. Klein I, Cornejo JC, Polakos NK, John B, Wuensch SA, Topham DJ, et al. Kupffer cell heterogeneity: functional properties of bone marrow-derived and sessile hepatic macrophages. Blood 2007;110:4077-4085.
5. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest 2005;115:56-65.
6. Imamura M, Ogawa T, Sasaguri Y, Chayama K, Ueno H. Suppression of macrophage infiltration inhibits activation of hepatic stellate cells and liver fibrogenesis in rats. Gastroenterology 2005;128:138-146.
7. Tacke F, Randolph GJ. Migratory fate and differentiation of blood monocyte subsets. Immunobiol 2006;211:609-618.
8. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, et al. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. J Clin Invest 2007;117:185-194.
9. Marra F, DeFranco R, Grappone C, Milani S, Pastacaldi S, Pinzani M, et al. Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. Am J Pathol 1998;152:423-430.
10. Shimizu Y, Murata H, Kashii Y, Hirano K, Kunitani H, Higuchi K, et al. CC-chemokine receptor 6 and its ligand macrophage inflammatory protein 3 alpha might be involved in the amplification of local necroinflammatory response in the liver. HEPATOLOGY 2001;34:311-319.
11. Tacke F, Ginhoux F, Jakubzick C, van Rooijen N, Merad M, Randolph GJ. Immature monocytes acquire antigens from other cells in the bone marrow and present them to T cells after maturing in the periphery. J Exp Med 2006;203:583-597.
12. Malato Y, Sander LE, Liedtke C, Al Masaoudi M, Tacke F, Trautwein C, et al. Hepatocyte-specific inhibitor-of-kappaB-kinase deletion triggers the innate immune response and promotes earlier cell proliferation during liver regeneration. HEPATOLOGY 2008;47:2036-2050.
13. Yagmur E, Weiskirchen R, Gressner AM, Trautwein C, Tacke F. Insulin resistance in liver cirrhosis is not associated with circulating retinol binding protein 4. Diabetes Care 2007;30:1168-1172.
14. Tacke F, Trautwein C, Zhao S, Andreeff M, Manns MP, Ganser A, et al. Quantification of hepatic thrombopoietin mRNA transcripts in patients with chronic liver diseases shows maintained gene expression in different etiologies of liver cirrhosis. Liver 2002;22:205-212.
15. Schwabe RF, Bataller R, Brenner DA. Human hepatic stellate cells express CCR5 and RANTES to induce proliferation and migration. Am J Physiol Gastrointest Liver Physiol 2003;285:949-958.
16. Tacke F, Marini FC III, Zhao S, McQueen T, Konopleva M, Ruvolo PP, et al. Expression of inducible Bcl-X(S) in myeloid leukemia: compensatory upregulation of Bcl-X(L) and Bcl-2 prevents apoptosis and chemosensitization. Cancer Biol Ther 2004;3:340-347.
17. Ginhoux F, Tacke F, Angeli V, Bogunovic M, Loubeau M, Dai XM, et al. Langerhans cells arise from monocytes in vivo. Nat Immunol 2006;7:265-273.
18. Borojevic R, Monteiro AN, Vinhas SA, Domont GB, Mourao PA, Emonard H, et al. Establishment of a continuous cell line from fibrotic schistosomal granulomas in mice livers. In Vitro Cell Dev Biol 1985;21:382-390.
19. Margis R, Borojevic R. Retinoid-mediated induction of the fat-storing phenotype in a liver connective tissue cell line (GRX). Biochim Biophys Acta 1989;1011:1-5.
20. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. Nat Immunol 2006;7:311-317.
21. Engel DR, Maurer J, Tittel A, Weisheit C, Cavler T, Schumak B, et al. CCR2 mediates homeostatic and inflammatory release of Gr1high monocytes from the bone marrow, but is dispensable for bladder infiltration in bacterial urinary tract infection. J Immunol 2008;181:5579-5586.
22. Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. J.Clin.Invest 2007;117:524-529.
23. Dunay IR, Damatta RA, Fux B, Presti R, Greco S, Colonna M et al. Gr1(+) Inflammatory Monocytes Are Required for Mucosal Resistance to the Pathogen Toxoplasma gondii. Immunity 2008;29:306-317.
24. Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity 2003;19:59-70.
25. Merad M, Manz MG, Karsunky H, Wagers A, Peters W, Charo I, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. Nat Immunol 2002;3:1135-1141.
26. Ravindran R, Rusch L, Itano A, Jenkins MK, McSorley SJ. CCR6-dependent recruitment of blood phagocytes is necessary for rapid CD4 T cell responses to local bacterial infection. Proc Natl Acad Sci U S A 2007;104:12075-12080.
27. Jakubzick C, Tacke F, Ginhoux F, Wagers AJ, van Rooijen N, Mack M, et al. Blood monocyte subsets differentially give rise to CD103+ and CD103- pulmonary dendritic cell populations. J Immunol 2008;180:3019-3027.
28. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat Med 2007;13:1324-1332.
29. De Minicis S, Seki E, Uchinami H, Kluwe J, Zhang Y, Brenner DA, et al. Gene expression profiles during hepatic stellate cell activation in culture and in vivo. Gastroenterology 2007;132:1937-1946.
30. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat Rev Immunol 2004;4:583-594.
31. Boring L, Gosling J, Chensue SW, Kunkel SL, Farese RV, Broxmeyer HE, et al. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. J Clin Invest 1997;100:2552-2561.
32. Leicester KL, Olynyk JK, Brunt EM, Britton RS, Bacon BR. Differential findings for CD14-positive hepatic monocytes/macrophages in primary biliary cirrhosis, chronic hepatitis C and nonalcoholic steatohepatitis. Liver Int 2006;26:559-565.
33. Panasiuk A, Zak J, Kasprzycka E, Janicka K, Prokopowicz D. Blood platelet and monocyte activations and relation to stages of liver cirrhosis. World J Gastroenterol 2005;11:2754-2758.
34. Marsillach J, Bertran N, Camps J, Ferre N, Riu F, Tous M, et al. The role of circulating monocyte chemoattractant protein-1 as a marker of hepatic inflammation in patients with chronic liver disease. Clin Biochem 2005;38:1138-1140.
35. Yamauchi K, Akbar SM, Horiike N, Michitaka K, Onji M. Increased serum levels of macrophage inflammatory protein-3alpha in hepatocellular carcinoma: relationship with clinical factors and prognostic importance during therapy. Int J Mol Med 2003;11:601-605.

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